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SOLID WASTE CELLULOSE DEGRADATION BY THERMOMONOSPORA CURVATA.(U)
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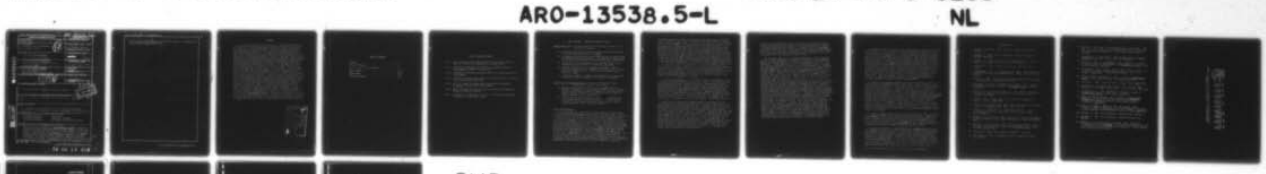

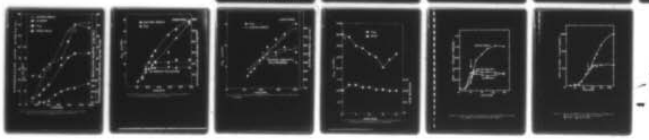

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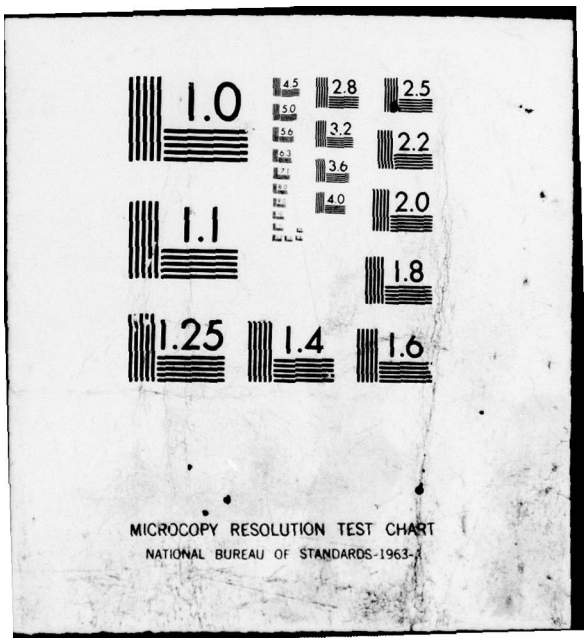
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18. SUPPLEMENTARY NOTES

The findings in this report are not to be construed as an official Department of the Army position, unless so designated by other authorized documents.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The cellulolytic potential of Thermomonospora curvata, a thermophilic actinomycete isolated from municipal solid waste compost, was evaluated. Studies on induction and repression of cellulase synthesis revealed that lactose, a potent inducer in Trichoderma, is a repressor in T. curvata, whereas cellobiose acts as inducer. Cellulase production is greatly influenced by Ca concentration and completely inhibited by low levels of pyridinium herbicides. T. curvata rapidly degrades a wide variety of

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20. (Abstract continued) solid waste cellulosic materials including some types of coated wrapping materials.

FORWARD

As estimated 4×10^{18} kilojoules of energy is stored on earth each year as plant material, providing an energy equivalent of 5×10^{4} barrels of oil per second (1). During photosynthesis, much of this energy is trapped in the anhydroglucose polymer, cellulose, and this cellulose constitutes our most abundant energy source (2). A large portion of this cellulose is wasted (more than 9×10^{8} metric tons annually in the United States alone) and this waste is projected to increase in the future (3). Beginning with the passage of the Solid Waste Disposal Act (Public Law 89-272), research efforts toward more effective means of disposal and bioconversion of waste cellulose to useful products have gained new economic and environmental importance. The high temperature composting of municipal refuse through the action of the indigenous microflora provides one method for volume reduction, elimination of pathogens, and conversion to a useful product. Since cellulose constitutes about 50% of the municipal refuse (largely in the form of waste paper), the rate of cellulose degradation during the composting process controls the efficiency of the overall process (4). Therefore, the study of those microorganisms responsible for cellulose degradation during composting is of prime importance in efforts to improve the rates of composting and the quality and stability of the finished product. Many of the available data on large scale municipal refuse composting have been obtained from studies at the Joint US Public Health Service-Tennessee Valley Authority Composting Project at Johnson City, Tenn. This pilot project, operated from 1967-71, provided an integrated evaluation of the microbiological, engineering, economic, and agricultural aspects of the composting process as a means of waste conversion to a usable agricultural product. The microbiological studies on cellulose degradation during composting as a rate-limiting factor revealed that *Thermomonospora curvata*, a thermophilic actinomycete, was the most actively cellulolytic of the flora isolated and tested (5). The following report describes the results from a study on the cellulolytic ability of *T. curvata* and some factors which influence growth and cellulase production.

TABLE OF CONTENTS

Forward	p.	2
Table of Contents		3
List of Illustrations and Tables		4
Body or Report		5- 8
Bibliography		9-10
Figures and Tables		11-17

LIST OF FIGURES AND TABLES

Fig. 1 Time course of extracellular cyclic AMP, C_x, protein and reducing sugars in cultures of T. curvata grown on ground cotton.

Fig. 2 Influence of 2-deoxy glucose (10^{-2}M) on C_x cellulase production by T. curvata when grown on ground cotton.

Fig. 3 Influence of alpha-methyl glucoside (10^{-2}M) on C_x cellulase production by T. curvata.

Fig. 4 Influence of lactose on C_x production and optical density in continuous culture operating at steady state.

Fig. 5 Effect of Diquat and Paraquat (20 µg herbicide/ml) on Growth of T. curvata in Slucose Mineral Salts Medium.

Fig. 6 Effect of Diquat (20 µg herbicide/ml) on Cellulase (C_1) Production. (Diquat was added at 10 hours.)

Table 1 Production of C_x cellulase as a function of limiting concentrations of cellobiose in the minimal medium.

BODY OF REPORT (OBJECTIVES AND MAIN RESULTS)

Research Objectives: The main objectives of the research described in this report were as follows:

a) to determine the effects which non-cellulosic soluble carbohydrates have on cellulase production by T. curvata;

b) to measure the effect which certain metals (Al, Ca, Fe, and Mg) have on cellulase production and activity. These metals are those present in the highest concentrations in municipal solid waste compost (6);

c) to purify the various components of the cellulase complex and to characterize their activities on a variety of substrates;

d) to measure the extent which T. curvata can degrade a range of substances commonly found in municipal refuse compost, such as waste paper and cardboard products, canning wastes, and agricultural cellulosic refuse;

e) to determine whether T. curvata could degrade any of the wide range of cellulosic plastics currently used in commercial packaging.

Significance of Research Objectives: The rational behind the research objectives stated above is as follows:

a) to define the cellulolytic potential of T. curvata, both as a dominant microorganism in the indigenous microflora of high-temperature composting, and further to determine whether pure cultures of this organism would be suitable for conversion of cellulosic wastes to usable substances such as soluble sugars;

b) to determine the optimal conditions for cellulase production and for cellulase activity in order to provide a meaningful basis for manipulation of the composting physical and chemical environment to achieve maximal rates of cellulose degradation.

Results and Discussion:

Cellulose degradation is catalzyed by an extracellular, multicomponent system which hydrolyzes the anhydroglucose polymer to soluble sugars, usually a mixture of glucose and cellobiose. Most of the study on the genetic control of cellulase production and the induction-repression of its synthesis has been carried out on Trichoderma species, and results have indicated that a variety of sugars can act as inducers of cellulase production at one concentration and as repressors at higher concentrations (7,8). However, little description has been made of the mechanism of this dual effect. In the present studies on T. curvata, the kinetics of cellulase repression by glucose appeared identical to those exhibited by the betaglucosidase system in Escherichia as described by Pastan and Perlman (9). Both phases of "catabolite repression" were apparent when cultures of T. curvata growing on cellulose were exposed to 10^{-2}M glucose. The transient phase occurred within 30 min of glucose addition and lasted for approximately 20 h; cellulase production again resumed after that time but at a rate about one-half that of the control (permanent repression). Cyclic adenosine monophosphate (cAMP) had been shown to act as an antagonist of catabolite

repression in Escherichia (10), and therefore, studies were made to determine the relationship of intracellular and extracellular levels of cAMP to cellulase production in T. curvata. Figure 1 illustrates the time course of extracellular cAMP, cellulase, protein, and reducing sugars accumulation in culture fluids of T. curvata grown on ground cotton fibers as the sole carbon and energy source. The rates of extracellular cAMP and cellulase accumulation were parallel over the entire 5-day growth period. However, the concentration of intracellular cAMP (data not included in this figure) decreased rapidly from about 7 picomoles per μg cell N at 10 h to about one picomole at 40 h with little increase for the remainder of the 5-day growth period. It is apparent from these data that the intracellular level of cAMP is decreased during cellulase production by excretion of the cyclic nucleotide into the medium when the level of free reducing sugars reaches detectable concentrations. In additional studies, exogenous cAMP (1 mM) did not reverse the catabolite repression; this lack of reversal is probably due to the impermeability of T. curvata to the cyclic nucleotide, indicated by apparent lack of ^{3}H-labelled cAMP uptake by the cells in preliminary experiments. The relationship of cAMP to cellulase synthesis regulation should be further explored: Kline and co-workers (11) have demonstrated that a variety of imidazole acetic acid and indole acetic acid derivatives can substitute for cAMP in regulation of catabolic enzymes. Such derivatives should be tested for their effect on cellulase regulation in T. curvata.

Studies were conducted to determine whether glucose or a metabolic product thereof was the active repressor in the celluase system. This was accomplished by replacing glucose by non-metabolizable analogs, 2-deoxyglucose (2-DG) and alpha-methylglucoside (MG). Neither of these compounds were utilizable as carbon and energy sources by T. curvata as shown by lack of growth on prolonged incubation. Figure 2 illustrates the effect of 10^{-2}M 2-DG on extracellular cellulase and protein accumulation in fluids of T. curvata cultures on ground cotton fibers. Complete repression of cellulase synthesis was achieved shortly after addition of the analog. This effect was similar but somewhat less marked in the case of MG (Fig. 3). In both cases, however, it was apparent that glucose and not a degradation product is the repressor of cellulase production.

Cellobiose, the predominant breakdown product of cellulase action on crystalline cellulose, has been demonstrated to be a potent repressor of cellulase synthesis in studies on both fungi (12) and bacteria (13). However, this disaccharide induced cellulase production in T. curvata, and while not as potent an inducer as crystalline cellulose, supported significant enzyme levels in continuous cultures as shown in Table 1. Conversely, lactose has long been known to be an excellent inducer of cellulase production in Trichoderma (12). However, in T. curvata, lactose was a potent repressor even at very low concentrations. Figure 4 illustrates this repressive effect of lactose in continuous culture under steady state conditions. When the culture of T. curvata gorwing on cellobiose was pulsed with a 4 μM concentration of lactose, cellulase production ceased immediately and did not resume until the lactose had been diluted out, despite the increased growth caused by lactose addition. These conflicting results indicate that induction of cellulase synthesis in T. curvata merits further study to ascertain the control mechanism.

In addition to catabolite repression by glucose, it was found that disaccharides also caused permanent repression of cellulase production, although this varied from one sugar to another. For example, growth of T. curvata on cellobiose "primed" the cells for maximal cellulase production after washing and transfer to cotton fibers, while growth of cells on maltose before transfer to cellulose caused permanent repression lasting for the duration of a 4-day incubation period. These results are shown in Fig. 4.

The permanent repression of cellulase production by maltose raised the question of starch degradation in compost (since maltose is the predominant product of starch hydrolysis) and its effect on the initiation of cellulose degradation. Starch comprises up to 6% of biodegradable material in compost (14) and constitutes more available source of soluble sugars; its degradation has been suggested (15) as an easily monitored criterion of composting rates. Although Bergey's Manual (16) describes T. curvata as non-amylolytic, our studies indicated that the actinomycete produced high levels of extracellular α-amylase (17). However, the α-amylase synthesis by T. curvata (and the products liberated by its action) apparently does not interfere with cellulase production. Further studies (18) on the products of the purified amylase demonstrated that maltose was not a product which accumulates from T. curvata α-amylase, rather, a mixture of tetrasaccharide and pentasaccharide was produced. These studies on the interrelationship of cellulase and amylase in T. curvata indicate that this organism is capable of degradation of both starch and cellulose and that the presence of starch in compost should not significantly deter cellulase synthesis, provided that large populations of other amylolytic microbes (which liberate maltose as the predominant starch hydrolysis products) are not established. Although thermophilic actinomycetes are the predominant organisms in the composting environment (2), the effect of major interactions of microbial populations at various stages of composting must be evaluated. The metals (Al, Ca, Fe and Mg) occurring in high concentrations in municipal refuse compost were studied as to their effects on cellulase synthesis, reaction rates, and thermal stability. These effects have been published (19) and need not be repeated here. However, the effects of these metals on highly purified individual components of the cellulase complex should be measured. These studies depend on further refinement of purification procedures for the components which include the cellobiohydrolyse (C_1), the endoglucanase (C_x), and the beta-glucosidase (cellobiase). To date, the C_1 activity appears to reside in a single protein with a molecular weight of about 6×10^4D; the C_x activity is divided between 3 distinct proteins with molecular weight in the range of $60\text{-}80 \times 10^3$D. The cellobiase appears to be exclusively intracellular and does not appear in the culture fluids.

It was apparent from the literature that a major potential source of inhibition of cellulolytic activity in compost was the widespread use of herbicides, particularly those which are water-soluble. A variety of these herbicides was tested as to their effect on T. curvata, but only diquat and paraquat (quaternary ammonium salts of 4,4'-bipyridyl) were found to be inhibitory. This inhibition has been reported by Brock and Stutzenberger (20) and shall be discussed only briefly here. Fig. 5 illustrates the rapid cessation of growth after the addition of diquat or paraquat (20 μg/ml) to T. curvata cultures growing in minimal medium. The inhibitory effect on cellulase production by herbicide was somewhat slower (Fig. 6) but no less complete. Wilkinson (21) has pointed out the fact that these herbicides accumulate to high levels in plant tissues and persist for long periods of time. For example, herbage from a pasture sprayed with paraquat (one lb/acre) had a 200 ppm concentration of paraquat (ten-fold that used in our studies) four weeks later; cattle excreted 7-42 ppm paraquat in their feces after grazing on that herbage (22). Such concentrations are well in excess of those needed for inhibition of growth and cellulase production by T. curvata, and could limit cellulose degradation in composting agricultural wastes.

The capacity of T. curvata to degrade a wide range of cellulosic substances usually found in municipal solid waste compost (such as paper, cardboard, plant, and canning wastes) has been described recently (23). The ability of the organism to degrade these substances is influenced by association of the cellulose with lignin, by coating of the paper, and by the grinding process. However, the stability of the extracellular cellulase is also a factor; although it does not undergo thermal denaturation at 52 C (the usual temperature for culturing T. curvata) cellulase activity in the culture fluids declines rapidly on prolonged incubation. Previous reports (24,25) have indicated that a variety of enzymes are regulated by proteolytic activity during carbon starvation. In preliminary studies on protease synthesis in T. curvata, we have found that this enzyme may serve a variety of functions. Thermophilic actinomycetes have proven to be an important source of proteases (26) and these enzymes may enable them in nature to eliminate competitive microbial populations by lysis (27). The role of a protease in the stability of extracellular cellulase has been suggested (28) and should be studied further in this regard.

In a previous progress report (for the period ending July 1, 1977), the ability of T. curvata to degrade some cellulosic plastics* was described. Coated cellophanes are widely used in the packaging industry and can be easily broken down by this thermophilic actinomycete, although cellulose acetate appears to be very resistant to enzymatic attack. T. curvata can use cellobiose octaacetate as a carbon and energy source, and perhaps growth on this soluble derivative will increase the ability of the organism to degrade cellulose acetate.

In summary, this research has demonstrated that T. curvata possesses cellulolytic potential which may be profitably exploited in a wide range of applications. Its thermophilic nature diminishes the cooling costs in industrial applications, facilitates solid-liquid separation operations, and prevents the survival and accumulation of most organisms pathogenic to man. We have recently obtained a number of T. curvata strains isolated from a variety of environments in both the United States and England. Of the strains available, selection will be made as to maximal cellulase production and stability, maximal temperature for growth, and minimum nutrient requirements. This strain selection, together with mutagenesis and metabolite gene regulation studies (11) already appear promising.

* All cellulosic plastics courtesy of Film Division, Olin Corporation.

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Cellobiose (µg/ml)	100	200	300	400		500		600	700
Dilution Rate	0.15	0.03	0.03	0.15	0.03	0.15	0.03	0.15	0.15
Cx (u/ml)	0.75	1.00	1.10	1.25	1.00	0.95	1.30	0.75	0.68
O.D. 610 nm	0.07	0.10	0.16	0.23	0.22	0.30	0.28	0.30	0.34
Extr. Protein (µg/ml)	98	15	20	50	90	90	55	85	105

Table 1. Production of C_x cellulase as a function of limiting concentrations of cellobiose in the minimal medium.

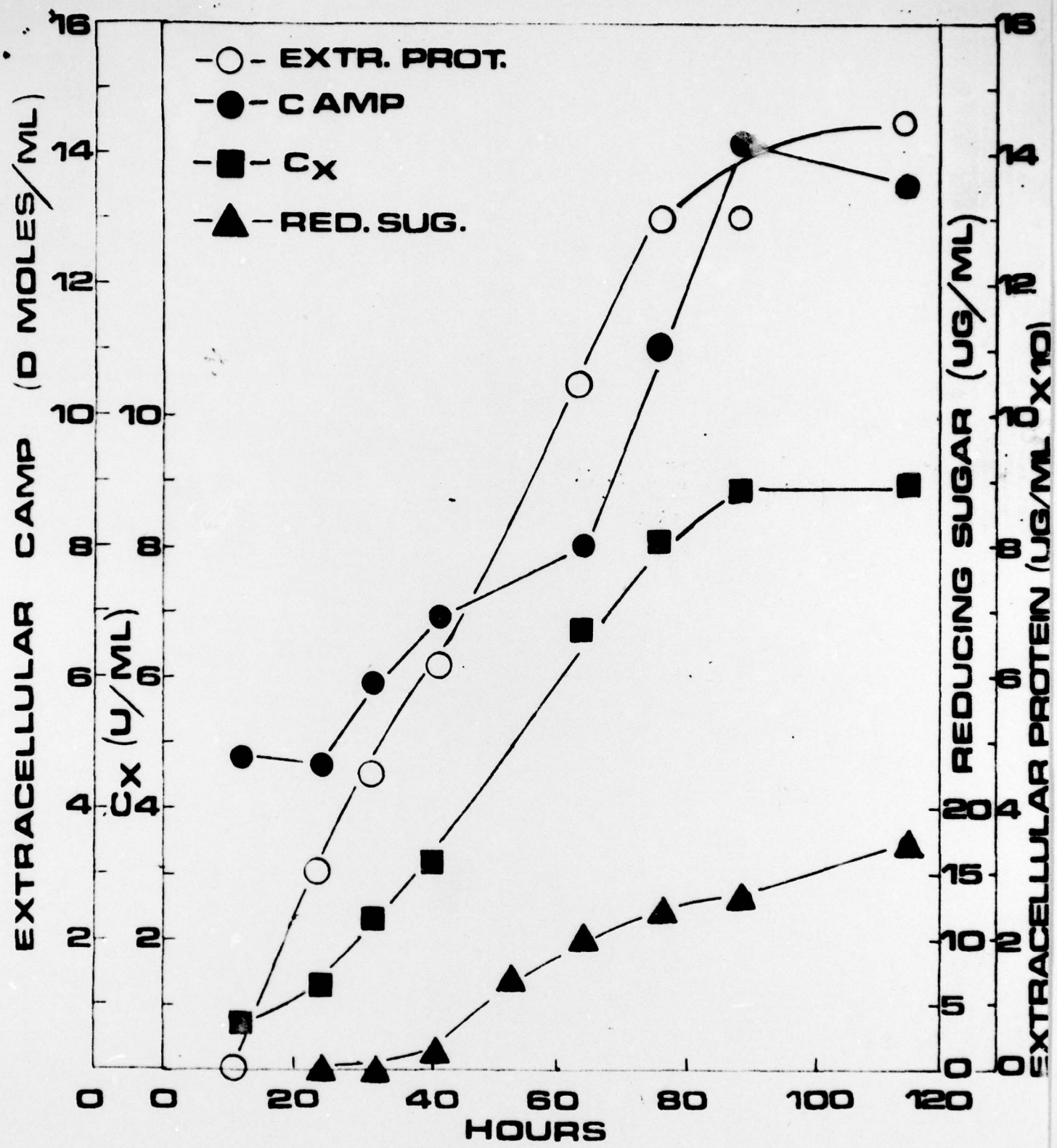

Fig. 1. Time course of extracellular cyclic AMP, C_x, protein and reducing sugars in cultures of T. curvata grown on ground cotton.

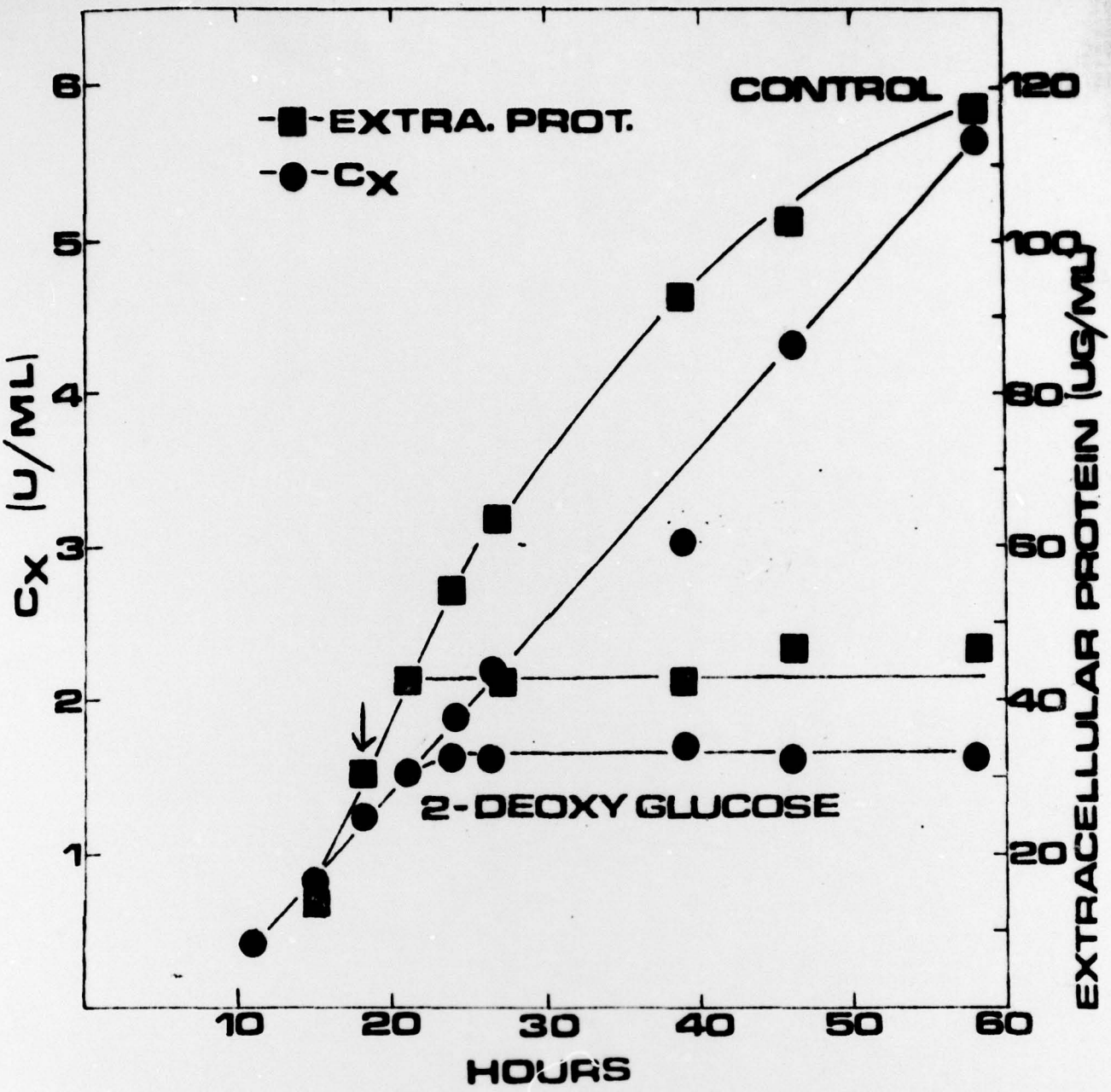

Fig.2. Influence of 2-deoxy glucose (10^{-2}M) on C_x cellulase production by T. curvata when grown on ground cotton.

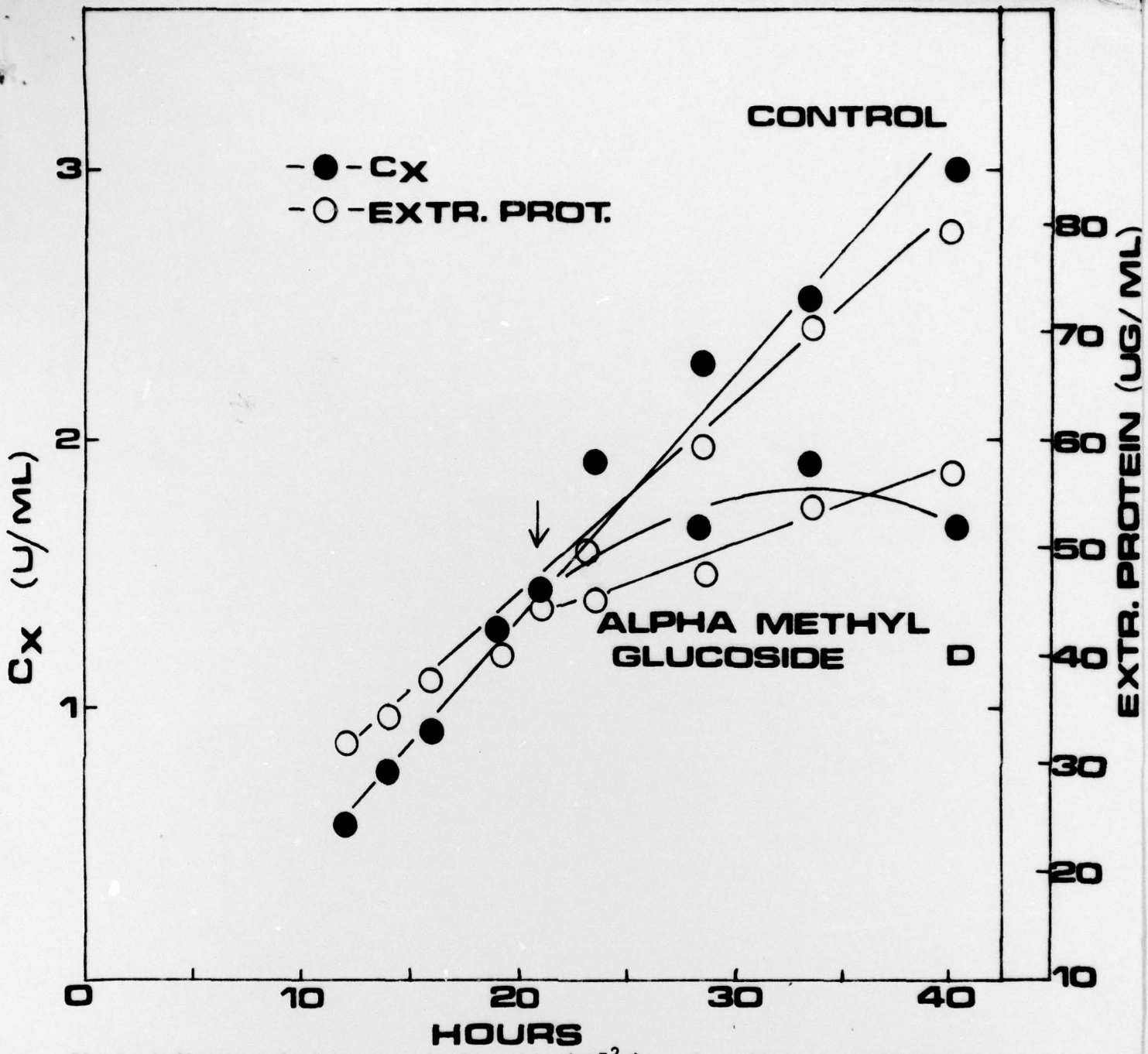

Fig.3. Influence of alpha-methyl glucoside (10^{-2}M) on C_x cellulase production by T. curvata.

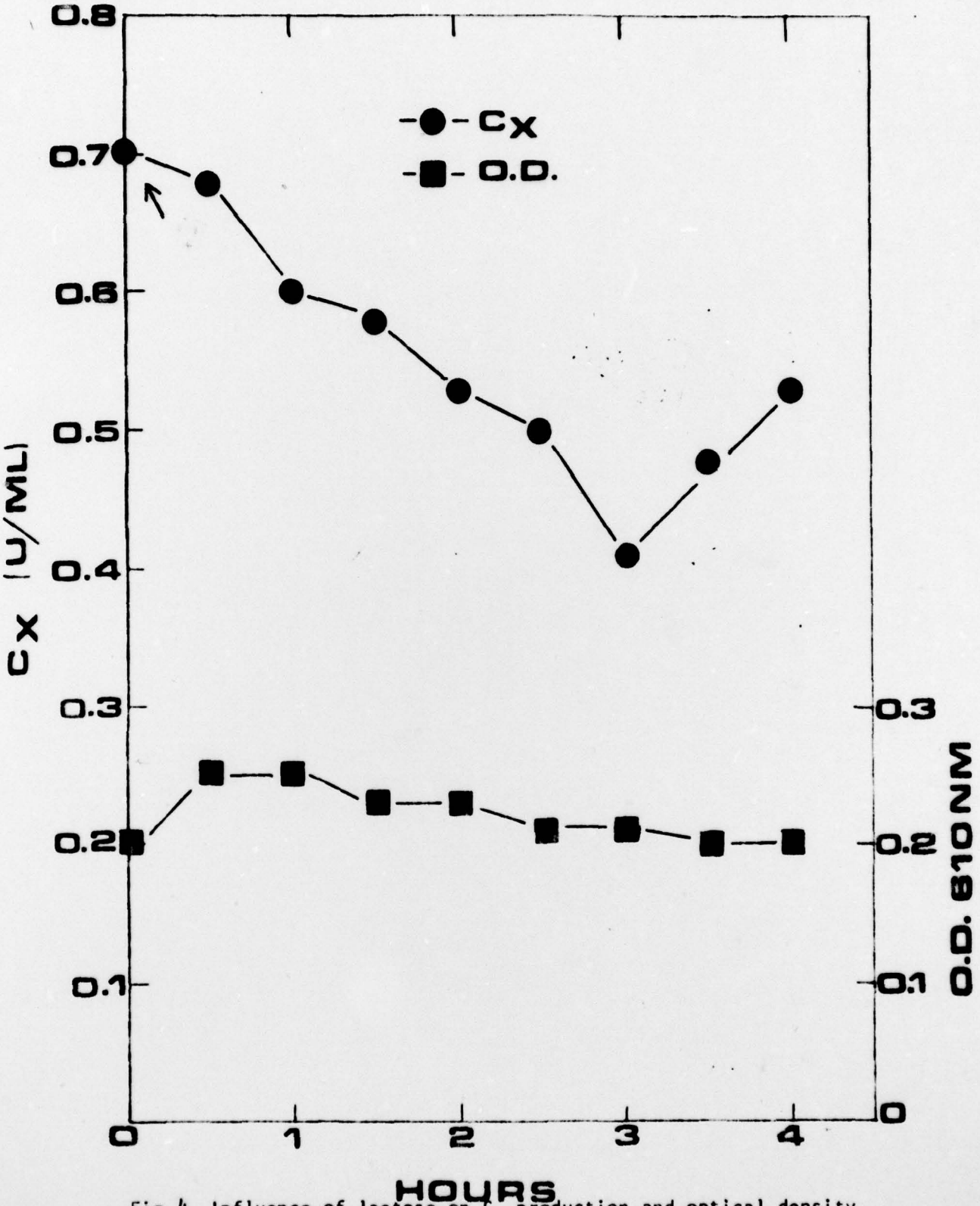

Fig. 4. Influence of lactose on C_x production and optical density in continuous culture operating at steady state.

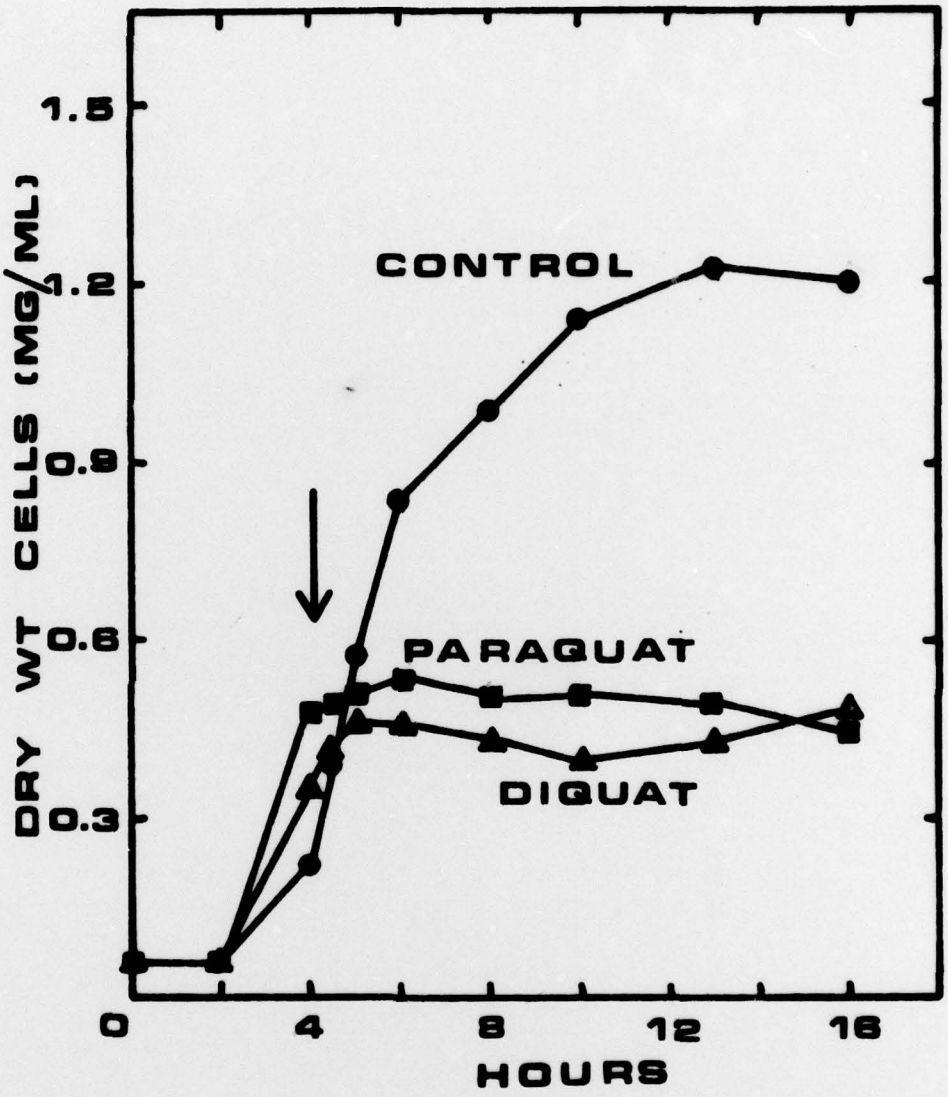

Figure 5. Effect of Diquat and Paraquat (20 µg herbicide/ml) on Growth of T. curvata in Glucose Mineral Salts Medium.

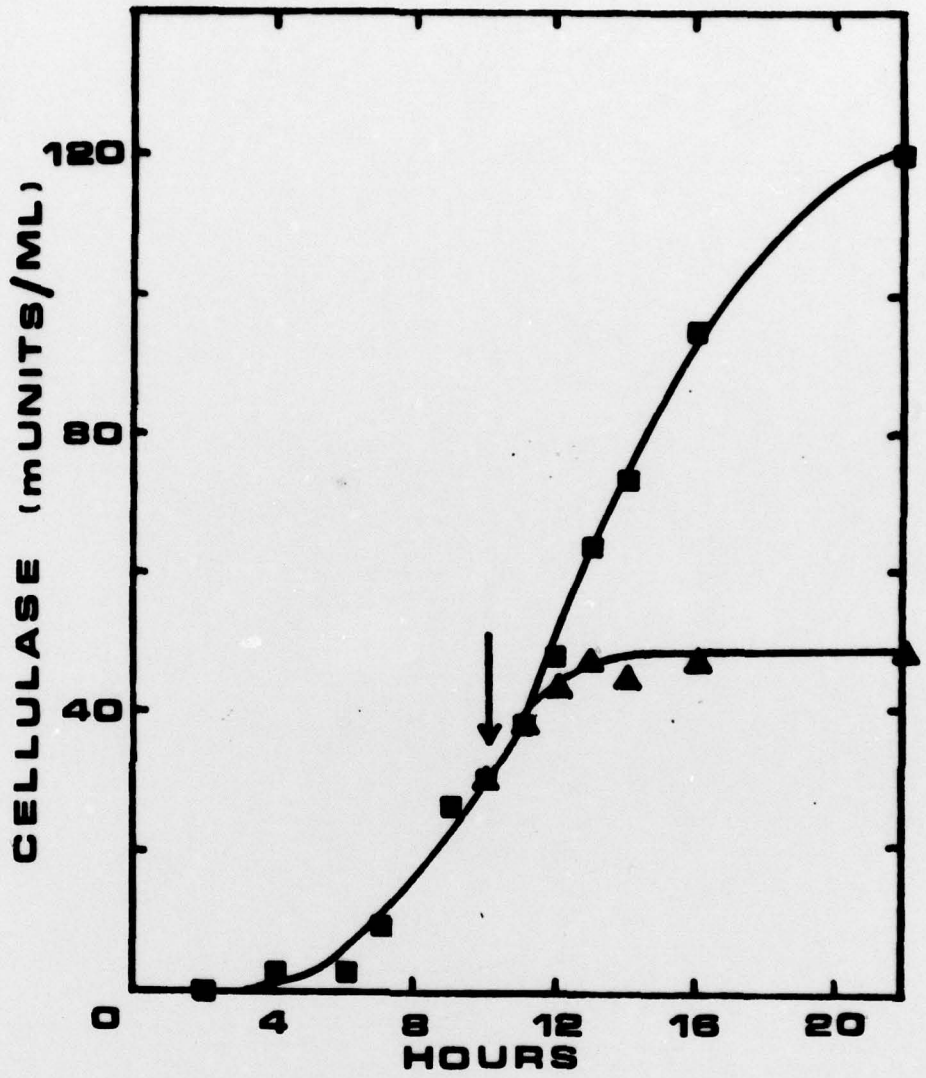

Figure 6. Effect of Diquat (20 µg herbicide/ml) on Cellulase (C_1) Production. (Diquat was added at 10 hours.)
■ Control. ▲ Diquat added.